Characterization of the Temperature Sensitive Phenotype of the A/Ann Arbor/6/60 Cold-Adapted Virus and Its Recombinants

BRIAN R. MURPHY, HUNNIEN F. MAASSAB, FRANK T. WOOD, JR., and ROBERT M. CHANOCK

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205, and Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan 48104

The A/Ann Arbor/6/60 cold-adapted (ca) donor virus had a 38°C shutoff temperature when tested in MDCK tissue culture. ca recombinant viruses bearing all six “internal genes” of the A/Ann Arbor/6/60 ca donor virus and the surface antigens of wild-type virus can manifest a 1,000-fold difference in plaquing efficiency at 38°C. These observations suggest that the A/Ann Arbor/6/60 ca donor genes that specify the temperature sensitive phenotype of the ca recombinants can undergo genetic modification during the production and passage of the recombinants. The NS gene in the ca recombinant virus could be inherited from either parent without influencing the level of temperature sensitivity of the ca recombinant.

Attenuation of new strains of influenza A virus can be accomplished by the transfer of attenuating genes (via genetic reassortment) from an attenuated donor virus to a new epidemic variant (1, 13). The influenza A/Ann Arbor/6/60 H2N2 cold-adapted (ca) virus is being evaluated as such a donor (1, 2, 5–8, 13). This mutant virus, which was produced by serial passage in primary chicken kidney tissue cultures at successively lower temperatures (5), replicates efficiently at 25°C, a temperature restrictive for growth of wild-type influenza A virus (5). The replication of the A/Ann Arbor/6/60 virus was also shown to be temperature sensitive (ts) (6, 16). Genes from the ca donor virus were transferred to a succession of new antigenic variants of influenza A virus, and several recombinant viruses bearing the ca and ts properties were found to be attenuated in animals and humans (2–4, 7, 8, 12, 14, 17).

One property of influenza A virus that is associated with attenuation is the level of temperature sensitivity of the virus in tissue culture (15). The level of temperature sensitivity of a ts virus can be quantitated by determining the in vitro shutoff temperature for plaque formation, i.e., the lowest temperature at which a 100-fold or greater reduction of virus titer is observed. The shutoff temperature of the A/Ann Arbor/6/60 ca parent virus was 37°C when tested in rhesus monkey kidney tissue culture and 38°C in primary chicken kidney culture (16). In addition, recombinants derived from the A/Ann Arbor/6/60 virus have been reported to have a 37, 38, 39, or greater than 39°C shutoff temperature (12, 17). Since the level of temperature sensitivity of a virus is an important determinant of attenuation, we sought to characterize this property of the A/Ann Arbor/6/60 parent virus and its recombinants in the readily available MDCK continuous cell line (15). In addition, the parental origin of the genes in each recombinant virus was determined so that the genetic basis for a variation in shutoff temperature of ca recombinants could be explored.

The techniques for the production of the recombinant viruses, for their genotyping, and for the determination of the efficiency of plaque formation in MDCK cultures have been described (2, 16). The genotype of the ca recombinant viruses had been previously determined (2).

The efficiency of plaque formation of wild-type and ca recombinant viruses is presented in Table 1. The A/Ann Arbor/6/60 ca parent virus had a 700-fold reduction of virus titer at 38°C. The A/Ann Arbor/6/60 ca parent virus was previously reported to have a 37°C shutoff temperature on rhesus monkey kidney cell monolayers (16). In simultaneous tests in rhesus monkey kidney and MDCK tissues, the ca parent virus had a 38°C shutoff temperature on both tissues if plaques were visualized by removing the overlay with subsequent hemadsorption. The two H3N2 wild-type viruses have a shutoff temperature of greater than 40°C, whereas that
<table>
<thead>
<tr>
<th>Influenza A virus</th>
<th>Cold-recombinant (CR) clone designation</th>
<th>Antigenic analysis</th>
<th>Parental origin of genes in recombinant virus at RNA segment (gene product)*</th>
<th>Log_{10} reduction of virus titer* (PFU/ml) at temp.</th>
<th>Shutoff temp* (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 (ND)</td>
<td>2 (ND)</td>
<td>3 (ND)</td>
</tr>
<tr>
<td>ca recombinant**</td>
<td>CR-6</td>
<td></td>
<td>AA</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Queensland/6/72</td>
<td>clone 0</td>
<td></td>
<td>H3N2</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Scotland/840/74</td>
<td>CR-18</td>
<td></td>
<td>H3N2</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Victoria/3/75</td>
<td>CR-29</td>
<td></td>
<td>H3N2</td>
<td>AA</td>
<td>wt</td>
</tr>
<tr>
<td>Alaska/6/77**</td>
<td>CR-29 clone 2</td>
<td></td>
<td>H3N2</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Alaska/6/77**</td>
<td>CR-31 clone 20</td>
<td></td>
<td>H3N2</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Hong Kong/123/77</td>
<td>CR-35</td>
<td></td>
<td>H3N2</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>clone 2</td>
<td></td>
<td>H1N1</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Ann Arbor/6/60 ca parent</td>
<td></td>
<td></td>
<td>H2N2</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Victoria/3/75 wild type</td>
<td></td>
<td></td>
<td>H3N2</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Alaska/6/77 wild type</td>
<td></td>
<td></td>
<td>H3N2</td>
<td>AA</td>
<td>AA</td>
</tr>
<tr>
<td>Hong Kong/123/77 wild type</td>
<td></td>
<td></td>
<td>H1N1</td>
<td>AA</td>
<td>AA</td>
</tr>
</tbody>
</table>

* ND, Not determined; HA, NA, NP, M, NS, gene products. AA, Gene derived from the A/Ann Arbor/6/60 ca parent; wt, gene derived from the wild-type parent.

* Average of two to seven tests in MDCK cultures. PFU, Plaque-forming units; NT, not tested.

* Shutoff temperature is defined as the lowest temperature at which a 100-fold or greater reduction of virus titer (plaque-forming units per milliliter) was observed.

** ca recombinant derived by mating the A/Ann Arbor/6/60 parent and the indicated wild-type virus.

*** The A/Alaska/6/77 wild-type and A/Ann Arbor/6/60 ca parents were the same in these two separate recombinations.
of the N1N1 wild-type virus was 40°C. The CR-6 (H3N2) and CR-35 (H1N1) ca recombinant viruses were approximately 100-fold more reduced in plaquing efficiency at 38°C than the A/Ann Arbor/6/60 ca parent, whereas the A/Alaska/6/77 CR-29 clone 2 recombinant was 20-fold less restricted. These differences were observed although each of the ca recombinants had received all six transferable genes from the A/Ann Arbor/6/60 parent. Two possible explanations for such observations are (i) that surface antigens derived from the wild-type virus can modify the temperature sensitive phenotype specified by the six transferable A/Ann Arbor/6/60 genes or (ii) that the temperature sensitive phenotype can be modified by mutations that occur during the production and passage of the recombinant virus.

To explore this latter possibility, the A/Ann Arbor/6/60 ca virus was mated with the A/Alaska/6/77 (H3N2) wild-type virus, and the genotype and shutoff temperature of six recombinant viruses were determined (CR-29 clone 2 and CR-31 clones 20, 2, 19, 3, and 10; Table 1). Four of the six ca recombinants received all of the six internal genes from the A/Ann Arbor/6/60 virus. The shutoff temperature of CR-31 clones 2 and 19 was like that of their A/Ann Arbor/6/60 ca parent. In contrast, CR-31 clone 20 was more restricted in plaque formation at 37 and 38°C than the ca parent virus (P < 0.005 at both temperatures, Student’s t test). The CR-29 clone 2 recombinant also differed significantly from its ca parent in its plaquing ability at 38°C, but in this case it was 20-fold less restricted. Four ca recombinant viruses received only five of the six transferable genes. Two of these, the A/Scotland/840/74 CR-18 clone 7 and the A/Alaska/6/77 CR-31 clone 10 recombinants, received a wild-type gene at the NS locus but retained the temperature sensitive phenotype characteristic of the A/Ann Arbor/6/60 parent. This suggests that the A/Ann Arbor/6/60 NS gene does not contribute significantly to the temperature sensitive phenotype of the ca viruses. Two other ca recombinants, the A/Victoria/3/75 CR-19 clone 0 and the A/Alaska/6/77 CR-31 clone 3 viruses, were less temperature sensitive than their parent ca virus and received a wild-type gene at the RNA-2 or M protein locus.

These results indicate that the recombinant viruses bearing all six internal genes of the A/Ann Arbor/6/60 ca donor virus and the surface antigens of a different influenza A virus can manifest a 1,000-fold difference in plaquing efficiency at 38°C. One explanation for these observations is that the A/Ann Arbor/6/60 ca donor genes that specify the temperature sensitive phenotype of the ca recombinants can undergo genetic modification during the production and passage of these viruses. Such modifications can either increase or decrease the level of temperature sensitivity of the recombinant. The occurrence of such modifying mutations has been inferred for ts recombinant viruses derived from the influenza A/Udorn/72-ts-1A2 donor virus (14b). In addition, a complex series of mutations, including extragenic suppressor mutations as well as intragenic mutations, was found to have modified the temperature sensitive phenotype of a ts virus that infected a susceptible child (14a). Another explanation for variation of the temperature sensitive phenotype in genomically comparable recombinants is that there existed in the suspensions of the cloned wild-type and ca parent viruses genes that specified the spectrum of temperature sensitive phenotypes observed.

The role the temperature sensitive phenotype plays in attenuation of the ca recombinant viruses derived from the A/Ann Arbor/6/60 ca parent is unclear. In contrast, ts genes appear to be responsible for attenuation of ts recombinant viruses derived from a chemically mutagenized virus (9-11). There was a good correlation between the shutoff temperature of a ts recombinant and its level of attenuation in humans (9). Three different viruses with a 39°C shutoff temperature produced febrile illness in over 10% of adult seronegative volunteers (9). ts recombinants exhibiting a 38°C shutoff temperature were generally satisfactorily attenuated in adults but not in seronegative children, whereas viruses with a 37°C shutoff temperature were satisfactorily attenuated in adults and children (1, 9). The ts mutations in these viruses were produced by chemical mutagenesis, whereas those present in the A/Ann Arbor/6/60 ca parent virus were selected during the serial passage at low temperature. With the former viruses, one might expect the ts genes to be the major determinants of attenuation, but with the latter virus, which was multiply passaged in tissue culture, one might expect to have additional non-ts mutations that alter virulence (13). If the major determinants of attenuation of the A/Ann Arbor/6/60 ca donor virus were its ts genes, then we would expect to see a significant variation in the level of attenuation in ca recombinants bearing different shutoff temperatures, but this has not been observed (15). For instance, the A/Alaska/6/77 CR-29 clone 2 ca recombinant, which has a 39°C shutoff temperature, did not induce febrile diseases in adults (13) or children (P. Wright, personal communication). These results suggest
that non-ts mutations of the A/Ann Arbor/6/60 parent virus play a primary role in attenuation of its recombinants in humans.

This work was supported in part by Public Health Service Contract N01 AI-72521 from the National Institutes of Health.

LITERATURE CITED


